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A critical role for Cadherin6B in regulating avian neural crest emigration

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Abstract

Neural crest cells originate in the dorsal neural tube but subsequently undergo an epithelial-to-mesenchymal transition (EMT), delaminate and migrate to diverse locations in the embryo where they contribute to a variety of derivatives. Cadherins are a family of cell-cell adhesion molecules expressed in a broad range of embryonic tissues, including the neural tube. In particular, *cadherin6B* (*Cad6B)* is expressed in the dorsal neural tube prior to neural crest emigration but is then repressed by the transcription factor *Snail2*, expressed by premigratory and early migrating cranial neural crest cells. To examine the role of *Cad6B* during neural crest migration, we have perturbed Cad6B protein levels in the cranial neural crest-forming region and have examined subsequent effects on emigration and migration. The results show that knock-down of Cad6B leads to premature neural crest cell emigration, whereas Cad6B overexpression disrupts migration. Our data reveal a novel role for *Cad6B* in controlling the proper timing of neural crest emigration and delamination from the neural tube of the avian embryo.

Keywords

Neural crest; cadherin; EMT; delamination; cell adhesion; (e)migration; chick embryo

INTRODUCTION

The vertebrate neural crest is a transient embryonic population of highly migratory cells that arises during neurulation, migrates extensively, and differentiates to create numerous distinct structures. In the avian embryo, precursors to neural crest cells (termed the premigratory neural crest) reside in the dorsal portion of the neural epithelium, the future central nervous system. Prior to neural crest migration, these precursors undergo changes in gene expression and morphology that facilitate an epithelial-to-mesenchymal transition (EMT) characterized by loss of the adherent, epithelial nature of the premigratory neural crest and subsequent acquisition of a mesenchymal, motile phenotype that exemplifies the migratory neural crest proper (Le Douarin and Kalcheim, 1999; Barembaum and Bronner-Fraser, 2005; Noden and Trainor, 2005; Sauka-Spengler and Bronner-Fraser, 2006).

EMTs are a hallmark of many distinct developmental processes, including mesoderm formation during gastrulation and tumor cell metastasis (Leptin et al., 1992; Ciruna et al.,

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1997; Thiery, 2002; Hemavathy et al., 2004). Members of the *Snail* family of transcription factors have been identified as having a central role in EMTs, acting as repressors that down-regulate expression of genes that help maintain an epithelial, polarized cell type. The molecular basis of *Snail*-mediated repression in various EMT events, both in the developing embryo and during tumorigenesis, has been well documented (Hemavathy et al., 2000; Nieto, 2002; Barrallo-Gimeno and Nieto, 2005; De Craene et al., 2005; Pérez-Mancera et al., 2005). Previous studies have demonstrated that one of the key molecular events underlying such EMTs is the down-regulation of the epithelial cadherin, *Ecadherin (E-cad)*. *In vitro* and *in vivo* experiments have revealed that this repression occurs through a direct interaction between Snail proteins and Snail binding sites (E boxes) in a palindromic position in the promoter region of *E-cad* (Giroldi et al., 1997; Cano et al., 2000; Bolós et al., 2003; Côme et al., 2004). Subsequent down-regulation of *E-cad* expression, at the transcriptional level by Snail proteins as well as post-transcriptionally through endocytosis and protein degradation (Palacios et al., 2005), results in the dismantling of adherens junctions and the loss of other "epithelial" characteristics in order to facilitate cell motility.

We have recently shown that the EMT that characterizes avian neural crest cell emigration from the dorsal neural tube involves the direct transcriptional repression of another Cadherin, *cadherin6B* (*Cad6B*), by Snail2. This represents the first reported direct target of Snail2 in the neural crest (Taneyhill et al., 2007) and defines a molecular mechanism for the decrease in *Cad6B* expression observed during neural crest EMT and emigration (Nakagawa and Takeichi, 1995, 1998). However, little is known about the function of *Cad6B* itself during neural crest EMT/emigration. To address this pertinent question, we have investigated the consequences of perturbing *Cad6B* expression in the dorsal neural tube of the chick embryo on subsequent neural crest emigration and migration. Our data reveal a previously unappreciated and critical role for *Cad6B* in temporally regulating neural crest emigration.

MATERIALS AND METHODS

Chicken embryo culture

Fertilized chicken eggs were obtained from AA Enterprises (Ramona, CA) and incubated on their sides at 38°C in humidified incubators (Lyon Electric Co., Chula Vista, CA). Embryos were staged according to the number of pairs of somites (somite stage (ss)).

Design and electroporation of Cad6B antisense morpholino

A 3' lissamine-labeled antisense *Cad6B* morpholino (MO), 5'-ACCAGAAGCAATGGTAAGTTCTCAT-3', was designed to target the *Cad6B* mRNA according to the manufacturer's criteria (GeneTools, LLC) in order to prevent translation of Cad6B protein. A standard lissamine-labeled MO (GeneTools, LLC) was utilized as a control, as well as a 5 base pair mismatch lissamine-labeled antisense *Cad6B* MO 5'-ACgAGAAcCAATcGTAAcTTCTgAT-3' (mutated bases are in lower case). MOs were introduced into the developing chick embryo at the required stage using a modified version of the electroporation technique (Itasaki et al., 1999). Briefly, the MO was injected at 250 μM into the lumen of the neural tube at the desired axial level and 2, 25 volt, 25 mSec pulses were applied across the embryo.

Overexpression of Cad6B in vivo

The full-length *Cad6B* gene was directionally cloned by PCR from a full-length *Cad6B* clone (Dr. Stephen Price) into the pCIG chick expression construct to produce pCIG-*Cad6B*. The clone was sequenced to confirm accuracy. The control (pCIG) or pCIG-*Cad6B* expression construct was introduced to the embryo at a concentration of 1.4 μg/μl, as described previously in the Materials and Methods for the electroporation of MOs.

Whole-mount in situ hybridization

Whole-mount *in situ* hybridizations were performed as previously described (Xu and Wilkinson, 1998; Taneyhill et al., 2007). Stained embryos were imaged in 70% glycerol using a camera mounted on a Zeiss Stemi SV11 microscope. Transverse-sections were obtained by cryostat-sectioning gelatin embedded embryos at 14 μm. Images were processed using Adobe Photoshop 7.0 (Adobe Systems).

Chick embryo and explant culture

Operated embryos were removed from the yolk sac using forceps and scissors, and a microscope was then used to count the number of somites. MO- or pCIG/pCIG-*Cad6B*-electroporated embryos were re-incubated for 30 minutes or 4 hours, respectively, prior to dissection. Dissection of appropriate tissue was performed using tungsten needles in PB-1 standard medium or Ringer's solution. Culturing of neural fold explants was performed by coating tissue culture dishes with a 1:100 dilution of recombinant fibronectin (BD Biosciences) and poly-Llysine (Sigma) in serum-free DMEM + N2 supplement (GIBCO-BRL) and mouthpipetting neural folds onto the coated dishes. Explants were cultured for 2.5 hours prior to fixation and immunostaining.

Immunohistochemistry

Immunohistochemical detection of Cadherin6B (DSHB, clone CCD6B-1; 1:100) was performed as previously described (Nakagawa and Takeichi, 1998), with an Alexa-Fluor 488 goat anti-mouse IgG secondary antibody (Molecular Probes, 1:500). Immunohistochemical detection of Cadherin7 (DHSB, clone CCD7-1; 1:100) was performed as previously described (Nakagawa and Takeichi, 1998), with an Alexa-Fluor 488 goat anti-mouse IgG_1 secondary antibody (Molecular Probes, 1:200). Immunohistochemical detection of N-cadherin (DHSB, clone NCD2; 1:50), phospho-histone H3 (1:500), and HNK-1 (1:100) were performed using similar methods. Embryos were washed, mounted, imaged and assessed for antibody efficacy as well as presence of MO or GFP. Embryos with good antibody staining and MO or GFP expression were selected and cryostat-sectioned (14 μm). Sections were DAPI-stained to identify individual nuclei prior to mounting and imaging. For detection and quantification of apoptosis in sections, an *in situ* cell death detection kit (‘TUNEL’ technology) was employed following the manufacturer's instructions (Roche). Samples were analyzed under the fluorescence microscope at either 594 nm or 488 nm.

Cell counts of Sox10-or Snail2-expressing cells

Embryos electroporated with control MO or *Cad6B* MO and hybridized with *Sox10* or *Snail2* antisense ribo-probes were imaged and subsequently cryostat-sectioned at 14 μm. Sections were stained with DAPI to enable the identification of individual nuclei and mounted for imaging. In midbrain regions where neural crest cell migration had commenced, 7-10 serial images were captured for at least 5 embryos that had MO localized to the dorsal neural tube. Every DAPI positive nuclei that was surrounded by cytoplasmic *Sox10* or *Snail2* staining in the migratory streams on both the electroporated and non-electroporated side was counted and recorded. The fold differences were averaged over the number of sections in which cells were counted, and the standard error of the mean was calculated and compared for embryos electroporated with either control MO or *Cad6B* MO. Significance of results was established using the unpaired Student's *t* test.

Cell counts for in vitro EMT/emigration explantation assay

The number of cells containing the morpholino (red) or expression construct (green) that have undergone EMT (i.e. mesenchymal in appearance) and have emigrated and migrated away from the neural fold explants was determined, using DAPI, phalloidin and/or vimentin staining

and phase contrast microscopy to identify individual cells for counting (Shoval et al., 2007; Taneyhill et al., 2007). This number was divided by the total number of cells that had emigrated away from the explant, and the resulting figure expressed as a percentage. Emigrating cells were counted in this manner from at least twenty-five explants over at least two independent experiments. Phalloidin staining was performed according to the manufacturer's instructions (Molecular Probes). Explants were also immunostained with an anti-vimentin antibody (DSHB, clone 40E-C; 1:50) using procedures described above.

RESULTS

In ovo knock-down of Cad6B results in premature neural crest cell emigration

To elucidate the role of Cad6B during neural crest emigration, we depleted Cad6B protein levels using an antisense MO targeting the 5' region of the *Cad6B* transcript. As controls for specificity, we performed similar experiments using either the GeneTools standard control MO or a 5 base pair mismatch *Cad6B* MO (5 bpmm MO). In all cases, Os were introduced into the neural tube by *in ovo* electroporation and the efficacy of knock-down was assessed in transverse sections of embryos immunostained for Cad6B protein. While Cad6B protein levels are unaffected under both control conditions (Fig. 1A and B, G and H, respectively), a marked decrease in immunodetectable Cad6B protein is observed in embryos treated with *Cad6B* MO (Fig. 1M and N). To further assess the specificity of the *Cad6B* MO, we examined the effects of other Cadherins expressed in the neural tube or neural crest, N-cadherin (Ncad) and Cadherin7 (Cad7), in embryos treated with either control, 5 bpmm or *Cad6B* MO. Ncad is normally expressed in apical adherens junctions found throughout the neuroepithelium (Takeichi and Nakagawa, 1995, 1998), while Cad7 is expressed exclusively on migrating neural crest cells (Takeichi and Nakagawa, 1995, 1998). We observed no change in the protein distribution of these other Cadherin family members in the presence of either type of control MO (Fig. 1C and D, I and J for Ncad; E and F, K and L for Cad7), or the *Cad6B* MO (Fig. 1O and P for Ncad; Q and R for Cad7), demonstrating the specificity of the *Cad6B* MO to deplete Cad6B but not other Cadherin proteins *in vivo*.

To address if knock-down of Cad6B alters neural crest EMT/emigration and subsequent migration, we examined the effects on expression of various known markers of premigratory or migrating neural crest cells. Chick embryos were electoporated with control or *Cad6B* MO into the midbrain region at the 2-4 somite stage (ss), re-incubated and then collected at the 8-10 ss, approximately 8 hrs later. Experimental embryos were assessed for MO electroporation efficacy, and only embryos exhibiting signal for MO in the midbrain region were subjected to further analysis. Embryos were processed for either whole-mount *in situ* hybridization for *Sox10*, *Snail2*, *FoxD3* or *rhoB*, or were immunostained with HNK-1 antibody, to assess potential changes in premigratory or migratory neural crest populations. In embryos treated with control MO, no changes were observed in either the pattern of migratory neural crest cells using the marker *Sox10* (n=8), or in the premigratory neural crest cell domain and the migratory pathway as assessed by *Snail2* (n=9) expression (Fig. 2A-D). Dynamic temporal changes in neural crest migration, however, were observed in embryos electroporated with *Cad6B* MO (Fig. 2H-K). The amount of *Sox10* and *Snail2* staining in neural crest cells as they emigrated away from the dorsal neural tube throughout the anterior-posterior (A/P) axis of the midbrain region on the electroporated side was consistently greater than that observed on the contralateral control side (Fig. 2I and J, arrows). This apparent increase in migration was observed in 8 out of 11 embryos hybridized with *Sox10* and 8 out of 11 embryos hybridized with *Snail2* (representative images; Fig. 2H-K). A similar increase in staining on the electroporated side was observed in embryos treated with *Cad6B* MO when hybridized for *FoxD3* (5 out of 6 embryos) and *rhoB* (5 out of 7 embryos) (Fig. 2L and M, respectively). Furthermore, HNK-1 immunostaining of *Cad6B* MO-treated embryos identified more staining

on the electroporated side when compared to the contralateral side and control embryos (4 out of 4 embryos; Fig. 2N).

To quantitate the increase in the number of migratory neural crest cells following knock-down of Cad6B, every DAPI positive nuclei that was surrounded by cytoplasmic *Sox10* or *Snail2* staining in the migratory streams through 7-10 serial sections on both the electroporated and non-electroporated side was counted and recorded in at least 5 embryos (representative images are shown in Fig. 2). The fold differences were averaged over the number of sections in which cells were counted, and the standard error of the mean was calculated and compared for embryos electroporated with either control MO or *Cad6B* MO. We found a statistically significant increase in *Sox10*-positive cells (1.32 +/−0.065) and *Snail2*-positive cells (1.44 +/− 0.077) on the electroporated sides of embryos treated with *Cad6B* MO compared to the contralateral, non-electroporated side ($P < 0.00001$; unpaired Student's *t* test), as evidenced by Figure 2I and J, respectively. In contrast, no change in cell number was observed with either migratory neural crest cell marker in embryos treated with control MO (*Sox10* = 1.03+/−0.020; *Snail2* = 1.03 +/− 0.014) (Fig. 2B and C, respectively). Taken together, our results suggest that depleted levels of Cad6B alter the timing at which neural crest cells leave the dorsal neural tube, such that the premature loss of Cad6B upon MO knock-down leads to early neural crest cell emigration and increased migration from the dorsal midbrain neural epithelium.

To further examine the effects of depleted levels of Cad6B on neural crest migration, we analyzed *Sox10* expression at both early and later time points post-electroporation of the *Cad6B* MO. As soon as 3 hours post-electroporation of the *Cad6B* MO, we detected *Sox10*-positive neural crest cells prematurely migrating from the midbrain dorsal neural tube (Fig. 3A and B, arrows). Moreover, as late as 20 hours post-electroporation, we also detected *Sox10*-expressing neural crest cells prematurely migrating into the periocular region on the *Cad6B* MO-transfected side compared to the control (Fig. 3C-E, arrows). In contrast, no alterations were noted at 3 and 20 hours post-electroporation in control MO-treated embryos (data not shown).

One potential explanation for the increase in neural crest cell migration exhibited by embryos treated with *Cad6B* MO could be an expansion of the premigratory neural crest cell domain. To address this possibility, we examined the expression pattern of the premigratory neural crest cell markers *Snail2*, *FoxD3* and *rhoB* in the dorsal neural tube in transverse sections of control MO- and *Cad6B* MO-treated embryos (Fig. 2B and C, E and F, I and J, and L and M). However, no change in the size of this region was detected. Other explanations for the earlier migration phenotype could include alterations in the rates of cell death and/or cell proliferation upon depletion of normal Cad6B levels. No apparent changes, however, were observed in the number of cells undergoing apoptosis (TUNEL stain), cell death (DAPI stain) or cell proliferation (phosphohistone H3 antibody staining), in *Cad6B* MO-treated embryos when compared to control MO-treated embryos (Fig. 3F). Rather, the premature migration is likely to resultfrom a loss of epithelial morphology that allows emigration of neural crest cells from the neural tube.

Knock-down of Cad6B enhances neural crest emigration/migration in vitro

To further test the hypothesis that Cad6B plays a temporal role in regulating neural crest emigration, we examined neural crest cell EMT and emigration using an *in vitro* assay in which neural folds are explanted and cultured (Shoval et al., 2007; Taneyhill et al., 2007). Effects on neural crest cell emigration and migration were then examined by phase microscopy (data not shown), DAPI (Fig.4B,D,F,H), phalloidin (an actin cytoskeleton marker; Fig. 4A and C) and vimentin (an intermediate filament marker that correlates with mesenchymal cells; Fig. 4E and G) staining after 2.5 hours of culture. This represents the time at which cells begin to exhibit features characteristic of emigrating neural crest cells that have just undergone EMT, such as

mesenchymal and fibroblast-like morphology (Fig. 4, arrows). Based on phalloidin, vimentin and DAPI staining, coupled with cell counting, our results show that fewer cells have undergone EMT, detached from the explant, emigrated, and become mesenchymal in the presence of control MO (Fig. 4A and B, E and F; n = 32 explants), when compared to cells containing *Cad6B* MO (Fig. 4C and D, G and H; n = 34 explants). The number of cells undergoing EMT and emigration when Cad6B protein levels were depleted was significantly increased over those in control cultures by approximately 3.5-fold ($P < 0.00001$; unpaired Student's *t* test) (Fig. 4I). Taken together with the *in ovo* findings, these results indicate that Cad6B plays a critical role in the initiation and timing of neural crest emigration in the chick embryo, as down-regulation of Cad6B is requisite for proper onset of neural crest emigration, both *in vitro* and *in vivo*.

In ovo overexpression of Cad6B results in disrupted neural crest cell (e)migration

To corroborate our *in ovo* knock-down data, we examined the effects of increasing Cad6B levels prior to neural crest EMT and emigration in the avian embryo. Chick embryos were electroporated with a control vector (pCIG) or a construct expressing full-length *Cad6B* in a bicistronic vector with a downstream IRES-GFP (pCIG-*Cad6B*). No change in Cad6B protein was observed in the presence of pCIG (Fig. 5A-C), whereas increased Cad6B levels were found throughout the dorsoventral axis of the neural tube when electroporated with pCIG-*Cad6B*, as evidenced by the double positive cells (yellow) expressing GFP and Cad6B (Fig. 5D-F).

We next addressed whether overexpression of Cad6B alters neural crest cell emigration and migration in the chick. Embryos were electroporated with pCIG or pCIG-*Cad6B* into the midbrain at the 2-4ss, re-incubated, and then collected approximately 8 hrs later at the 8-10ss. We then performed whole-mount *in situ* hybridization on embryos for *Sox10*, *Snail2*, *FoxD3* and *rhoB* or immunostaining for HNK-1 to monitor possible changes in neural crest emigration and migration (Fig. 6). Electroporation of the control vector resulted in no changes in the timing or number of migrating neural crest cells by *Sox10* (n=11), *Snail2* (n=14), *FoxD3* (n=6) or *rhoB* (n=5) expression or HNK-1 immunostaining (n=4) (Fig. 6A-G). In contrast, overexpression of Cad6B disrupted neural crest cell migration, manifested as a reduction in the overall extent of neural crest cell migration on the electroporated side compared to the contralateral control side. This was clearly visible throughout the A/P axis in transverse sections showing *Sox10* (9 out of 11), *Snail2* (12 out of 13), *FoxD3* (7 out of 8) and *rhoB* (4 out of 6) expression and HNK-1 (5 out of 5) immunostaining (Fig. 6H-N, arrows). Furthermore, neural crest cells in these embryos tended to stay closely associated with the neural tube and, those that were able to exit the neural tube, appeared to cluster (Fig. 6I,J,L,M; arrow). In some embryos, overexpression of Cad6B caused a number of cells to enter the lumen of the neural tube (Fig. 6I,J,L-N; arrowheads), an abnormal location and phenotype not observed in control electroporated embryos. Interestingly, this observation is similar to one previously seen when Ncad or Cad7 was ectopically expressed throughout the chick neural epithelium (Nakagawa and Takeichi, 1998), suggesting that increased levels of Cadherins generally affect proper neural crest cell emigration and migration. In addition, in some embryos overexpressing Cad6B, we observed striking alterations in neural tube morphology that were not found in embryos electroporated with the pCIG control construct. In keeping with the phenotype observed at 8 hours, embryos examined 20 hours post-electroporation of pCIG-*Cad6B* exhibit *Sox10*-positive cells adjacent to/associated with the neural tube (Fig. 7A and B), a phenotype not observed in embryos electroporated with the control pCIG construct (data not shown).

The apparent decrease in the ability of neural crest cell migration observed upon overexpression of Cad6B could be due to an alteration in the size of the premigratory neural crest domain. To test this possibility, we analyzed *Sox10*, *Snail2*, *FoxD3* and *rhoB* expression as premigratory neural crest cell markers in sections through electroporated embryos (Fig. 6I,J,L,M). No

changes, however, were noted in the size of the premigratory neural crest domain, as determined by counting the number of *Snail2*-positive cells in the dorsal neural tube region (treated and contralateral control side) of embryos electroporated with either pCIG or pCIG-*Cad6B*. In addition, elevated levels of Cad6B did not affect apoptosis (TUNEL), cell death (DAPI) or cell proliferation (phospho-histone H3) in treated embryos (Fig.7C). Interestingly, however, cells present in the lumen of the neural tube of embryos electroporated with the Cad6B expression construct were TUNEL positive, suggesting they were apoptotic (Fig.7C, arrows). Collectively, our data suggest that elevated levels of Cad6B lead to a decrease in the ability of neural crest cells to emigrate, and subsequently migrate away, from the dorsal neural tube.

We also assessed the effects of elevated Cad6B on emigration *in vitro* by explanting and culturing neural folds electroporated and expressing pCIG or pCIG-*Cad6B.* owever, under *in vitro* conditions, we observed no effects on neural crest emigration and migration when assessing cell morphology through phase microscopy and staining for phalloidin (Fig. 8A,C), DAPI (Fig. 8B,D,F,H) and vimentin (Fig. 8E,G). At 2.5 hrs, control and experimental cultures were indistinguishable from each other in the absence (Fig. 8A and B, E and F; n = 25) or presence (Fig. 8C and D, G and H; n = 25) of elevated Cad6B protein, with no statistical significant differences (Fig. 8I).

DISCUSSION

Cadherins, in conjunction with members of the catenin family of intracellular proteins, are the main components of cellular adherens junctions (Pla et al., 2001; Wheelock and Johnson, 2003; Lien et al., 2006). Localized to the apical surface of the cell, these junctions contribute to overall epithelial polarity by assisting in the formation of cortical actin cables surrounding the cell. Besides serving a fundamental role in mediating calcium-dependent cell-cell adhesion, Cadherins also function to modulate different intracellular signaling processes by integrating signals from the extracellullar matrix to cytoplasmic components of the cell. For example, cleavage of the cytoplasmic tail of Ncad releases a C-terminal product that functions in downstream transcriptional events, ultimately resulting in BMP-mediated neural crest delamination in avian embryos (Shoval et al., 2007). In addition, the cytoplasmic domain of E-cad has been shown to be a regulator of mammary epithelial morphogenesis (Delmas et al., 1999).

Various Cadherins are expressed during formation of the neural crest and its derivatives. In avian embryos, there are initially high levels of Cad6B and Ncad in neural crest precursor cells located in the dorsal neural tube (Duband et al., 1988; Akitaya and Bronner-Fraser, 1992; Nakagawa and Takeichi, 1995). In contrast, E-cad is expressed primarily in the non-neural ectoderm, with much lower levels found in the neural plate (Hatta and Takeichi, 1986). Prior to EMT and neural crest emigration, Ncad, and then slightly later Cad6B, are both down-regulated in the dorsal neural tube (Nakagawa and Takeichi, 1995; Liu and Jessell, 1998; Sela-Donenfeld and Kalcheim, 1999; Pla et al., 2001; Shoval et al., 2007; Taneyhill et al., 2007), with a concomitant increase in Cad7 and Cadherin11 expression on migrating crest cells, presumably to facilitate transient cell-cell interactions as neural crest cells migrate away from the neural tube to their final destinations in the embryo (Nakagawa and Takeichi, 1995; Nakagawa and Takeichi, 1998; Kulesa and Fraser, 2000; Borchers et al., 2001). Upon homing and differentiation, neural crest cells subsequently express other Cadherins depending upon the embryonic derivative they are fated to become. For instance, melanocytes found in the dermis, basement membranes and hair bulb express E-cad, while Ncad becomes up-regulated in aggregating neural crest cells prior to the formation of both the dorsal root and sympathetic ganglia (Pla et al., 2001), and during the patterning of the neural crest-derived cardiac outflow tract in the mouse (Luo et al., 2006).

Previous studies have investigated the role of some of the Cadherins expressed during neural crest emigration. Inactivation of *Ncad* or the use of calcium ion chelators in the chick results in premature neural crest emigration (Newgreen and Gooday, 1985; Akitaya and Bronner-Fraser, 1992; Bronner-Fraser et al., 1992; Pla et al., 2001), whereas overexpression of *Ncad* or *Cad7* in the chick dorsal neural tube suppresses neural crest EMT and migration, particularly along the dorsolateral pathway (Nakagawa and Takeichi, 1998). We have recently identified a direct regulatory relationship between another Cadherin, *Cad6B*, and Snail2, a transcriptional repressor expressed in the premigratory neural crest cell population (Taneyhill et al., 2007). This regulatory relationship, coupled with the temporal and spatial expression pattern of Cad6B, suggests that Cad6B might play an important role during neural crest EMT and emigration. To directly assess the role of Cad6B during neural crest EMT and emigration, we used a combined loss-of-function and gain-of-function approach. MO-mediated protein knock-down demonstrates that Cad6B plays an important temporal role in regulating neural crest emigration, such that its loss leads to precocious neural crest EMT and earlier emigration of neural crest cells away from the dorsal neural tube. This effect is specific to depletion of Cad6B, as we observe no change in the protein distribution of other Cadherins, such as Ncad and Cad7, in *Cad6B* MO-treated embryos. *In ovo*, we observe a statistically significant 1.4-fold increase in the number of migrating neural crest cells in embryos with depleted levels of Cad6B. This is all the more remarkable in light of the intrinsic mosaic nature of MO transfections in the embryo, in that not all cells actually receive the MO0 and consequently reduce Cad6B protein levels. Furthermore, this premature emigration can be observed at multiple time points after introduction of *Cad6B* MO into the embryo, manifested as an increase in the number of migratory crest cells in the periphery of the embryo. The precocious migration is not due to changes in the rates of cell proliferation, cell death or apoptosis of the premigratory or migratory neural crest cell population, nor to changes in the timing of neural crest induction, but rather is due to an effect on the timing of neural crest cell delamination from the dorsal neural tube.

These *in vivo* findings were corroborated by examining effects on neural crest emigration using an explantation assay in which neural folds were electroporated with either control or *Cad6B* MO and cultured *in vitro*. This *in vitro* assay, coupled with staining for appropriate molecular markers, allows one to accurately assess the mesenchymal morphology of individual cells that have just undergone EMT and are emigrating and migrating. Under these conditions, knock-down of Cad6B results in a statistically significantly 3.5-fold increase in neural crest emigration and the production of migratory, mesenchymal cells. This knock-down phenotype is quite striking, as alterations in the level of just one molecule involved in cell-cell adhesion and EMT led to significant changes in the numbers of emigrating neural crest cells *in vitro*. This finding further underscores the critical regulatory relationship between Snail2 and *Cad6B* during neural crest emigration: down-regulation of *Cad6B* must occur for neural crest cells to exit the neural tube, and this is achieved, in part, by Snail2 repression. Our current study suggests that the repression of *Cad6B* requires tight temporal control, as premature down-regulation of *Cad6B* leads to the earlier onset of neural crest emigration. Thus, the molecular changes underlying neural crest emigration associated with *Cad6B* repression must be regulated in the proper spatiotemporal manner in order to ensure subsequent neural crest cell migration in the avian embryo.

Further substantiating the Cad6B knock-down phenotype, overexpression of Cad6B *in vivo* results in decreased or disrupted neural crest cell emigration and migration. This effect is observed at varying time points post-electroporation, as we consistently observe cells aggregating by the dorsal neural tube and find a decrease in the total number of neural crest cells migrating into the periphery. Furthermore, overexpression of Cad6B does not alter cell proliferation or cell death rates in either the neural tube or migratory neural crest cells, and neural crest induction appears unaffected in these embryos. *Cad6B* expression is normally restricted to the dorsal neural tube, a site that correlates with that of the premigratory neural

crest. As such, the forced expression of Cad6B in more ventral and thus ectopic regions sometimes affects the architecture of the neural tube as well, as observed in some of our electroporation experiments, perhaps via alterations in the shape and/or organization of the neural epithelial cells. Having high levels of Cad6B in the premigratory neural crest, however, creates a potentially problematic situation, as neural crest cells undergoing EMT/emigration must down-regulate *Cad6B* to facilitate proper migration, and these precursor cells may be unable to effectively do so in the presence of continuous *Cad6B* expression. Furthermore, we often observed cells in the lumen of the neural tube, a phenotype reminiscent of that seen when Ncad or Cad7 are overexpressed (Nakagawa and Takeichi, 1998). Similar to the conclusions reached by this previous study, here it is likely that cells enter the lumen as a result of 1) Cad6B localization throughout the apical and lateral surfaces of the neuroepithelium, with the loss of restricted Cad6B protein distribution dorsally, and/or 2) the loss of potential directionality in neural crest cell emigration as cells attempt to exit the neural tube. It should be noted that the penetrance of this phenotype is likely due to the mosaic nature of the electroporation technique.

To investigate the mechanism by which Cad6B controls neural crest cell emigration and subsequent migration, we performed *in vitro* explantation assays with neural folds overexpressing Cad6B. These results reveal that premigratory neural crest cells in the dorsal neural tube are capable of emigrating from the explants, even in the presence of ectopic Cad6B, implying that Cad6B does not have a global effect on cell motility. The discrepancy observed in our *in vivo* (where cells fail to migrate properly) and *in vitro* (where cells migrate normally) Cad6B overexpression assays is likely due to the differences between the more complex/non-permissive extracellular matrix in the embryo and the uniform/permissive substrate of the *in vitro* culture dish. Thus, our data support a hypothesis in which changes in cell-cell adhesion are precluding normal neural crest cell migration in embryos overexpressing Cad6B, and that loss of Cad6B-mediated adhesion is critical to permit timely emigration of neural crest cells from the dorsal neural tube in the embryo. As such, this apparent increase in cell-cell adhesion among those emigrating neural crest cells overexpressing Cad6B in the embryo proper can sometimes result in a ‘traffic jam’ with cells ‘backing up’ with some spilling into the lumen of the neural tube. Inevitably, these cells undergo apoptosis because of their improper location in the embryo. It is also possible that those neural crest cells that emigrate and exit the neural tube (with or without altered levels of Cad6B) may subsequently adhere and become ‘trapped’ by those cells situated proximal to the neural tube, thus precluding further migration.

Our results show that the controlled temporal and spatial expression of Cadherins in the neural tube prior to and during neural crest cell migration appears to be important, at least in part, for proper neural crest development. Ncad is broadly expressed throughout the neural epithelium prior to the onset of neural crest emigration, and it is down-regulated in the dorsal premigratory neural crest cell region just prior to emigration in a BMP-mediated fashion (Nakagawa and Takeichi, 1995; Shoval et al., 2007). Concurrently, Cad6B is up-regulated in the premigratory region, perhaps initially to distinguish the presumptive dorsal premigratory/progenitor neural crest cell region from the surrounding neural epithelial cells (Liu and Jessell, 1998; Sela-Donenfeld and Kalcheim, 1999). The localization of Cad6B to the premigratory neural crest region and its subsequent down-regulation appears to be requisite for controlling the precise timing of emigration and the onset of proper neural crest cell migration in a Snail2-dependent manner (Taneyhill et al., 2007; this study). Taken together, our results reveal a previously unappreciated role for Cad6B in regulating the timing of neural crest emigration and the exit of neural crest cells from the dorsal neural tube in the cranial region of the avian embryo.

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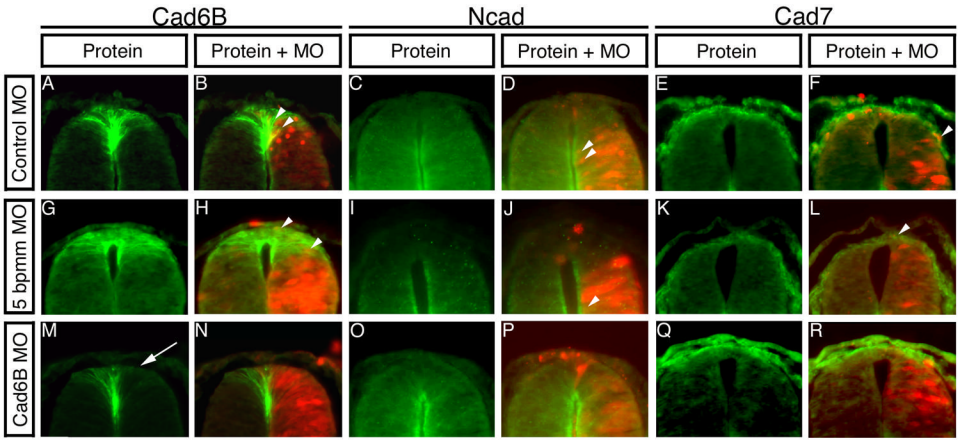

Figure 1. Introduction of *Cad6B* MO reduces the amount of Cad6B protein in the dorsal neural tube, but does not affect the protein distribution of Ncad or Cad7

Transverse sections through the trunk of a chick embryo with 21-23 somites at the axial level +4 and −1 somite. Embryos have been stained for Cad6B, Ncad or Cad7 protein (green) and electroporated with either a lissaminated (red) GeneTools standard control morpholino (Control MO), 5 base pair mismatch control MO specific to Cad6B (5 bpmm MO), or *Cad6B* morpholino (Cad6B MO). For each condition, the pair of panels shown are a representative section taken from at least 5 different embryos analyzed. Upper panels (A-F) show a section analyzed for either Cad6B, Ncad or Cad7 that have been transfected with the control MO (B,D,F, respectively). Arrowheads identify cells that are both red and green (yellow) and thus positive for control MO and either Cad6B (B), Ncad (D) or Cad7 (F) protein. Middle row of panels (G-L) show a section analyzed for either Cad6B, Ncad or Cad7 that have been transfected with the 5 bpmm MO (H,J,L, respectively). Arrowheads identify cells that are both red and green (yellow) and thus positive for 5 bpmm MO and either Cad6B (H), Ncad (J) or Cad7 (L) protein. Lower panels (M-R) show a section analyzed for either Cad6B, Ncad or Cad7 that have been transfected with the *Cad6B* MO (N,P,R, respectively). Treatment with *Cad6B* MO results solely in a reduction in the size of the Cad6B domain in the dorsal neural tube compared to the contralateral control side (N, arrow), and, consequently, very few (if any) red and green (yellow) cells present. No changes in Ncad nor Cad7 protein distribution is observed upon depletion of Cad6B, as evidenced by the presence of red and green (yellow) cells that are positive for Cad6B MO and Ncad (P) or Cad7 (R) protein.

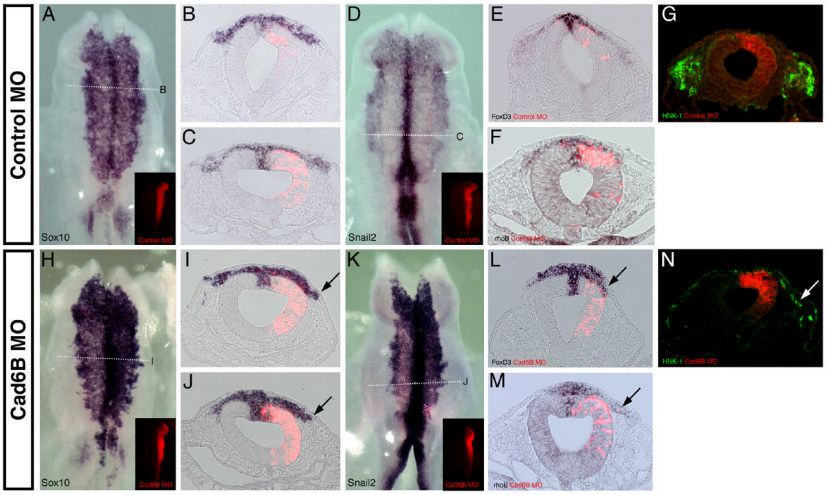

Figure 2. Decreased levels of Cad6B result in an increase in neural crest cell migration

Electroporations of either control MO (A-G) or *Cad6B* MO (H-N) into the midbrain region of chick embryos at the 2-4ss; embryos were allowed to develop until the 8-9ss. Whole-mount *in situ* hybridizations with antisense ribo-probes for *Sox10* (A and H) or *Snail2* (D and K) and indicated sections ((B and I) and (C and J), respectively). Transverse sections of embryos processed by *in situ* hybridization with antisense ribo-probes for *FoxD3* (E and L) and *rhoB* (F and M) or by immunohistochemistry for HNK-1 (G and N). Note that Panel N is slightly more caudal than Panel G, illustrating the premature migration phenotype observed in embryos treated with *Cad6B* MO. In all panels, the electroporated side is on the right, as indicated by the inset or overlaid lissamine (red) fluorescence staining showing cells that have received the MO. Arrows identify an increase in the amount of staining when compared to the contralateral control side (I and J, and L-N), as well as to control MO-containing embryos (B and C, and E-G). Treatment with control MO had no effect on neural crest cell migration at any axial level examined.

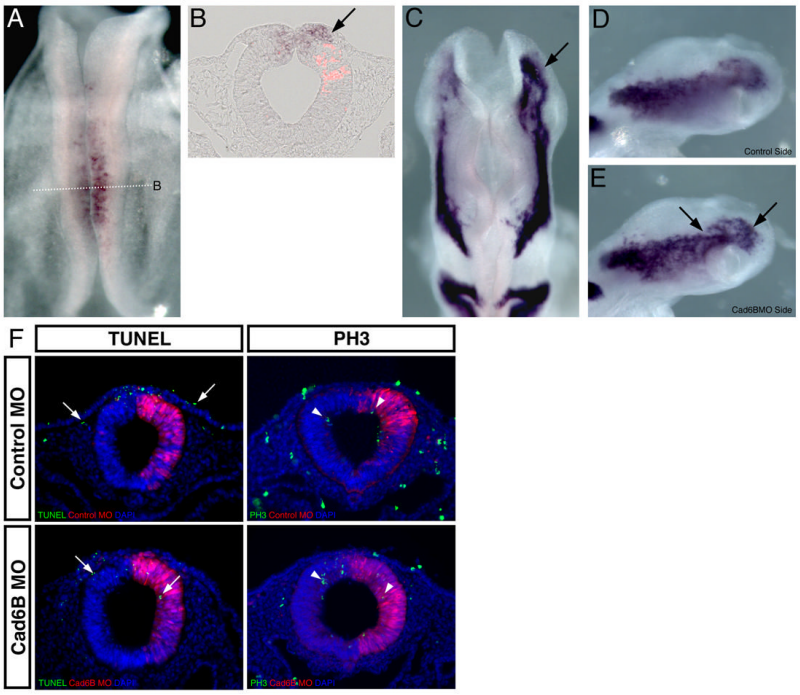

Figure 3. Introduction of the *Cad6B* MO results in premature migration of cranial neural crest cells but does not effect cell death or cell proliferation

Electroporations of *Cad6B* MO into the chick midbrain region of the embryo at the 2-4ss followed by either 3 hours (A and B), 20 hours (C-E) or 8 hours (F) of incubation. In panels A-C and F, the electroporated side is on the right; panels D and E are labeled appropriately. Whole-mount *in situ* hybridizations with antisense ribo-probes for *Sox10* (A and C-E) and indicated section (B). Black arrows identify *Sox10*-positive, prematurely migrating neural crest cells as they leave the midbrain (B), and at later developmental stages migrating into the periocular region (C and E) that are not observed on the contralateral control side (D). (F) Transverse sections of embryos in which a TUNEL assay has been performed or which have been immunostained for phospho-histone H3 (PH3) after electroporation with either control MO or *Cad6B* MO do not identify any changes between MO-treated (red) and control sides. Cells undergoing apoptosis exhibit granular punctate green stain in the TUNEL assay (white arrows), while the number of proliferating cells assessed by phospho-histone H3 staining (green, white arrowheads) remains unchanged in the presence or absence of either MO. Individual cells are stained blue with DAPI.

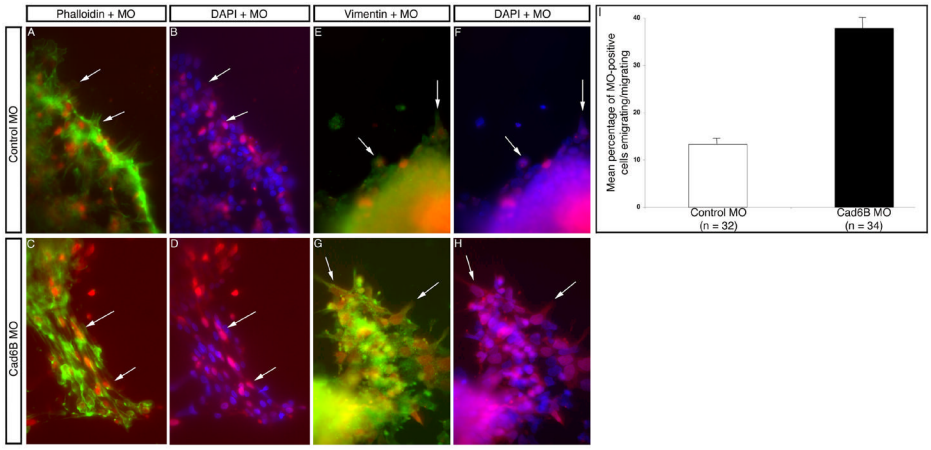

Figure 4. Knock-down of Cad6B results in an increase in neural crest EMT/emigration *in vitro*

Explantation and 2.5 hour culture of chick dorsal neural folds (containing the premigratory neural crest) electroporated *in vivo* with control MO or *Cad6B* MO (red) and stained for phalloidin (green, A and C), vimentin (green, E and G), and DAPI (blue; B,D,F,H). Phalloidin staining shows the presence of extended actin-filled processes (arrows), most prominent in emigrating and migrating neural crest cells from explants taken from embryos electroporated with *Cad6B* MO compared to control MO (compare A and C). In addition, emigrating and migrating cells are positive for vimentin, a marker of intermediate filaments and mesenchymal cells (E and G, arrows). Fewer vimentin-positive (mesenchymal) cells are observed emigrating and/or migrating in explants taken from embryos electroporated with control MO versus *Cad6B* MO (E and G, respectively), and this result was confirmed by DAPI staining of the same explants (B and D, F and H). (I) Numerical graph showing standard error of the mean of explantation results. Cell counting and statistical analysis identifies a significant increase in the number of neural crest cells undergoing EMT/emigration in the presence of *Cad6B* MO when compared to control MO ($P < 0.00001$; unpaired Student's *t* test), such that *Cad6B* MO treatment results in the production of more mesenchymal cells.

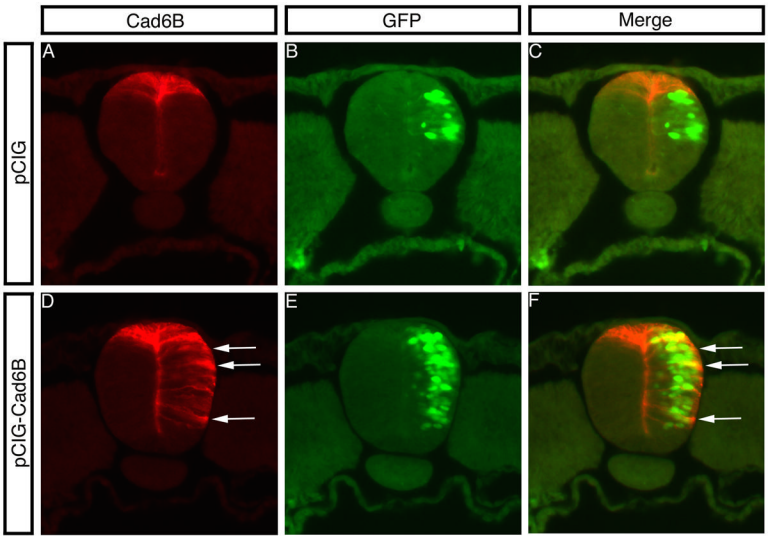

Figure 5. Introduction of the *Cad6B* expression construct results in elevated levels of Cad6B protein throughout the dorsoventral axis of the neural tube

Transverse sections through the trunk of a chick embryo with 21-23 somites at the axial level +4 and −1 somite. Embryos were electroporated with a control expression construct (pCIG) or a *Cad6B* expression construct (pCIG-*Cad6B*) containing an IRES-GFP to mark transfected cells (GFP, green) and incubated for 8 hours prior to fixation and staining for Cad6B protein (red). Upper panels (A-C) show a representative section from at least 4 different embryos that have been transfected with the control expression construct. No ectopic Cad6B protein is visible in the neural tube, as evidenced by the absence of any new cells that are both red and green (yellow) outside of the dorsal expression domain of Cad6B. Lower panels (D-F) show a representative section from at least 4 different embryos that have been transfected with the Cad6B expression construct. Expression of this construct results in elevated levels of Cad6B protein as well as an increase in the ventral distribution of Cad6B protein in the neural tube when compared to the contralateral control side (D, arrows). This results in the presence of multiple red and green (yellow) cells that are double-positive for the expression construct and Cad6B protein (F, arrows).

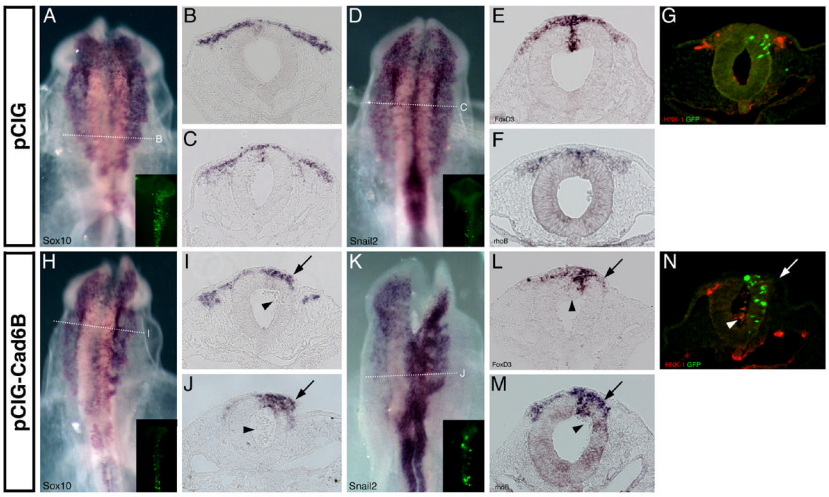

Figure 6. Elevated levels of Cad6B protein disrupt the migration of neural crest cells

Electroporations of either pCIG (A-G) or pCIG-*Cad6B* (H-N) into the midbrain region of chick embryos at the 2-4ss; embryos were allowed to develop until the 8-9ss. Whole-mount *in situ* hybridization with antisense ribo-probes for *Sox10* (A and H) or *Snail2* (D and K) and indicated sections ((B and I) and (C and J), respectively). Transverse sections of embryos processed by *in situ* hybridization for *FoxD3* (E and L) and *rhoB* (F and M), or immunohistochemistry with HNK-1 (G and N). In all panels, the electroporated side is on the right, as indicated by the inset GFP image (green fluorescence) showing cells that have received the transfected DNA construct. Arrows identify migratory neural crest cells that have failed to properly migrate away and thus aggregate adjacent to the dorsal neural tube, when compared to the contralateral control side (I and J, and L-N), as well as to pCIG electroporated control embryos (B and C and E-G). Arrowheads identify cells that have entered into the lumen of the neural tube (I and J and L-N).

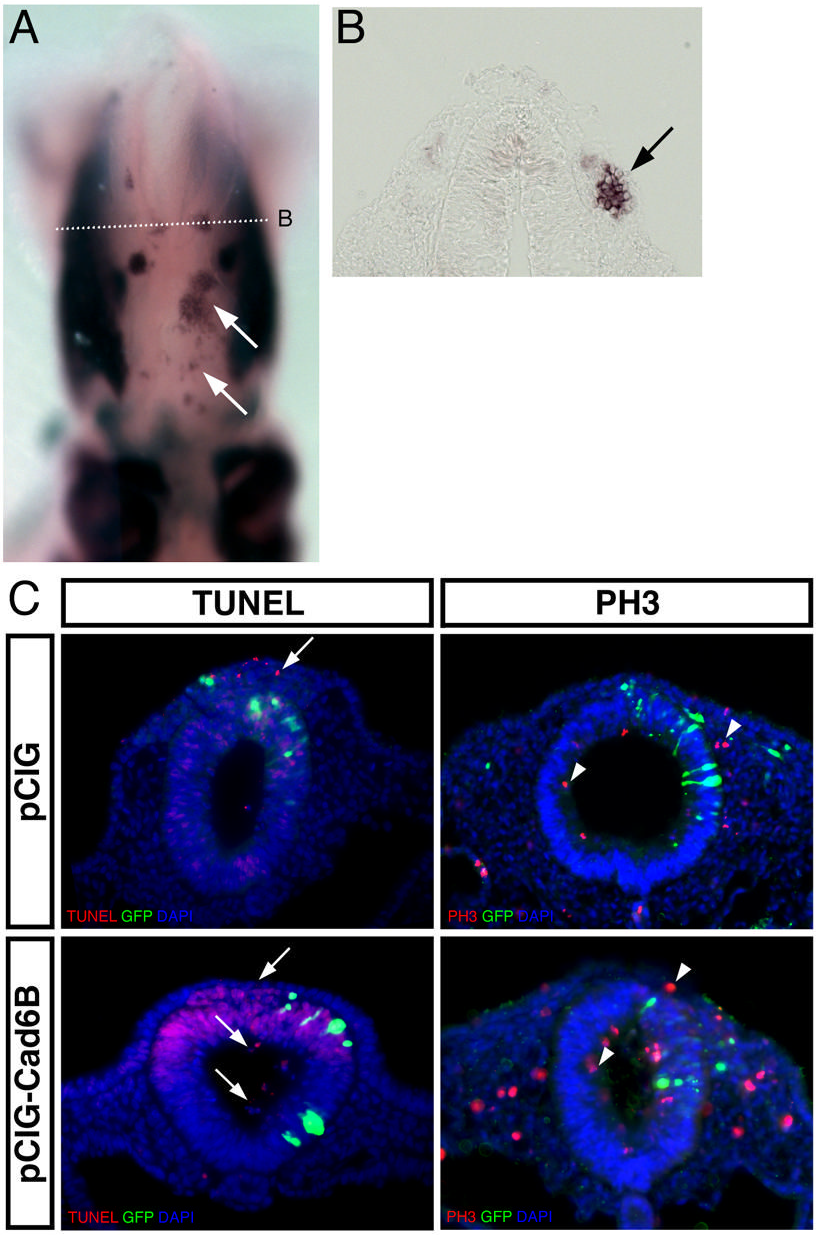

Figure 7. Neural crest cells expressing elevated levels of Cad6B remain associated with the neural tube with no change in cell proliferation nor apoptosis in the neural tube

Electroporations of the pCIG-*Cad6B* expression construct (green) into the chick midbrain region of the embryo at the 2-4ss followed by either 20 hours (A and B) or 8 hours (C) of incubation. In all panels, the electroporated side is on the right. Whole-mount *in situ* hybridization for *Sox10* (A), and indicated section (B), identifies a number of *Sox10*-positive cells adjacent to the neural tube (arrows), a phenotype not detected in control embryos. (C) Transverse sections of embryos in which a TUNEL assay has been performed or which have been immunostained for phospho-histone H3 (PH3) (both red) after electroporation with either pCIG or pCIG-*Cad6B* do not identify any changes between transfected (GFP, green) and control sides in the neural epithelium. Cells present in the lumen of the neural tube of embryos overexpressing Cad6B are undergoing apoptosis and exhibit granular punctate red stain (white arrows). The number of proliferating cells assessed by phosphohistone H3 staining (red, white

arrowheads) remains unchanged in the presence or absence of elevated levels of Cad6B. Individual cells are stained blue with DAPI.

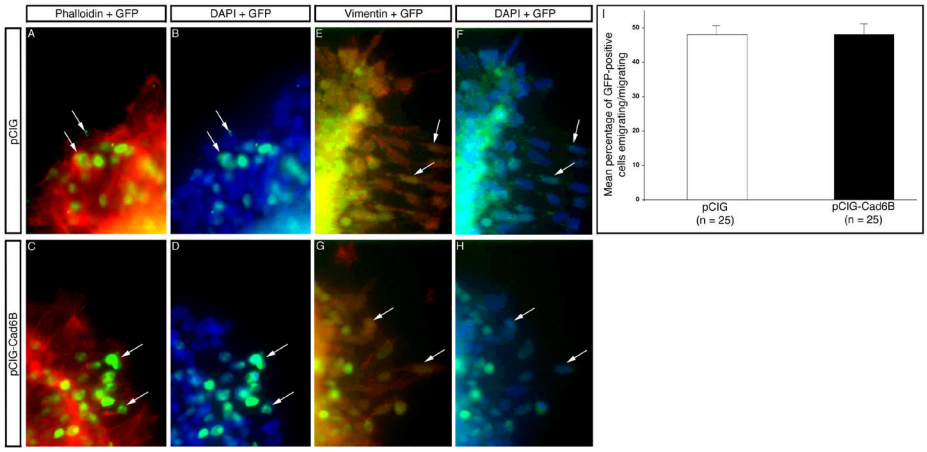

Figure 8. Overexpression of Cad6B does not alter neural crest emigration and migration *in vitro*

Explantation and 2.5 hour culture of chick dorsal neural folds (containing the premigratory neural crest) electroporated *in vivo* with either pCIG or pCIG-*Cad6B* (green) and stained for phalloidin (red, A and C), vimentin (red, E and G), and DAPI (blue; B,D,F,H). Phalloidin staining shows the presence of extended actin-filled processes in emigrating and migrating neural crest cells from explants taken from embryos electroporated with pCIG or pCIG-*Cad6B* (A and C, arrows). In addition, emigrating and migrating cells in both cultures are positive for vimentin, a marker of intermediate filaments and mesenchymal cells (E and G, arrows). (I) Numerical graph showing standard error of the mean of explantation results. Statistical analysis does not identify any significant difference between the number of neural crest cells emigrating/migrating in neural fold explant cultures taken from embryos electroporated with pCIG (48.0%) or pCIG-*Cad6B* (48.2%).